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# Association of gut microbial dysbiosis with disease severity, response to therapy and disease outcomes in Indian patients with COVID-19

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## Abstract

**Background** Severe coronavirus disease 2019 (COVID-19) is associated with systemic hyper-inflammation. An adaptive interaction between gut microbiota and host immune systems is important for intestinal homeostasis and systemic immune regulation. The association of gut microbial composition and functions with COVID-19 disease severity is sparse, especially in India. We analysed faecal microbial diversity and abundances in a cohort of Indian COVID-19 patients to identify key signatures in the gut microbial ecology in patients with severe COVID-19 disease as well as in response to different therapies. The composition of the gut microbiome was characterized using 16S rRNA gene sequences of genomic DNA extracted from faecal samples of 52 COVID-19 patients. Metabolic pathways across the groups were predicted using PICRUSt2. All statistical analyses were done using Vegan in the R environment. Plasma cytokine abundance at recruitment was measured in a multiplex assay.

**Results** The gut microbiome composition of mild and severe patients was found to be significantly different. Immunomodulatory commensals, viz. Lachnospiraceae family members and *Bifidobacteria* producing butyrate and short-chain fatty acids (SCFAs), were under represented in patients with severe COVID-19, with an increased abundance of opportunistic pathogens like *Eggerthella*. The higher abundance of *Lachnoclostridium* in severe disease was reduced in response to convalescent plasma therapy. Specific microbial genera showed distinctive trends in enriched metabolic pathways, strong correlations with blood plasma cytokine levels, and associative link to disease outcomes.

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**Conclusion** Our study indicates that, along with SARS-CoV-2, a dysbiotic gut microbial community may also play an important role in COVID-19 severity through modulation of host immune responses.

**Keywords** COVID-19, Gut microbiome, Dysbiosis, Cytokine, Convalescent plasma

## Introduction

The novel coronavirus SARS-CoV-2 infection that led to the coronavirus disease of 2019 or COVID-19, led to a pandemic and a devastating public health crisis [1, 2]. With time, emerging variants of the virus have resulted in successive waves of community transmission worldwide [3, 4], causing close to 630 million infections that have led to approximately 6.5 million deaths till date. The unfavourable outcomes encountered in a fraction of patients mostly follow an acute respiratory distress syndrome (ARDS), which in turn follows systemic hyper-inflammation with a cytokine storm [2, 5]. Despite a lot of studies on systemic hyper-inflammation and its link to severe respiratory disease in some patients, the heterogenous immunopathology of severe COVID-19 and the indeterminacy of susceptibility to severe disease remain enigmatic [6–9]. Systemic immunosuppression using corticosteroids has been proven to be the most successful therapy [10, 11]. Anti-cytokine therapy targeting interleukin-6 is also effective in some, but not in all severe COVID-19 patients [12, 13]. Antiviral therapies using small molecules as well as combinations of antibodies have also shown limited efficacy [14–16]. Thus, meticulous charting of the systemic hyper-inflammation and the probable host-intrinsic factors playing a differential role in driving susceptibility to severity are of major importance. Moreover, a lot of variation in disease susceptibility as well as disease outcomes in different parts of the world in different cohorts of patients perhaps also points to demographic and environmental factors that shape individual host responses [17].

A crucial dimension of human heterogeneity in terms of disease risk and resilience is attributed to the commensal microbiota in different body sites [18]. The microbial ecologies of the human body have been explored in great detail in myriad noncommunicable and communicable diseases [19, 20]. With relevance to communicable infections, involvement of the gut microbiome in susceptibility to infections has been documented in bacterial infections like Salmonellosis, parasitic infections like malaria, as well as viral infections like influenza and respiratory syncytial virus [21–24]. In this context, the gut microbial influence on systemic immune mechanisms has been explored in detail in different contexts [25]. Moreover, significant interest is also driven by the putative microbiome-gut-lung axis [26]. Efforts have already been put in to explore the associative link between gut microbial dysbiosis and risk-resilience balance in the context of COVID-19 [27–29].

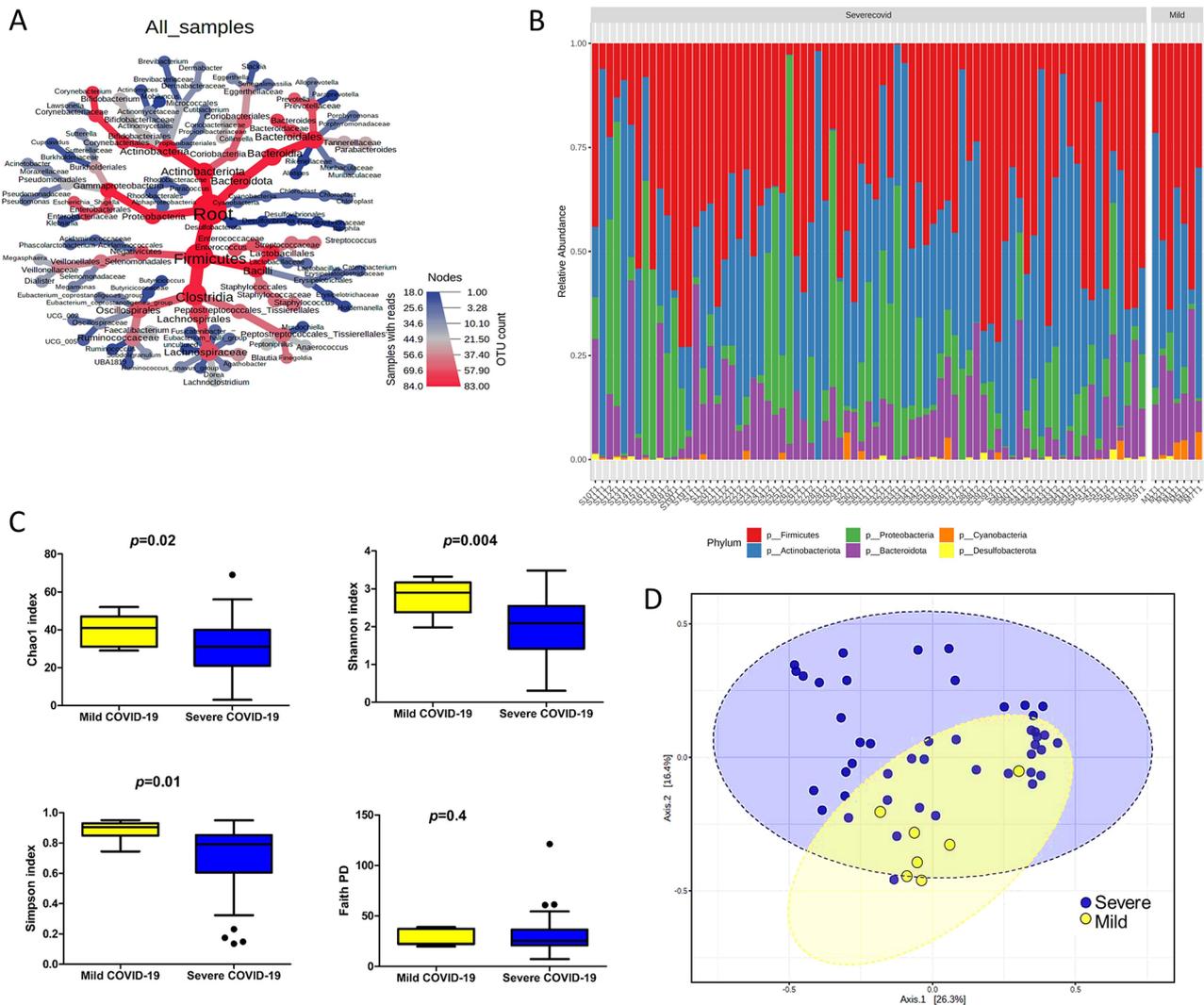
In the present study, we analysed faecal microbiota richness, diversity, and abundance in a cohort of Indian COVID-19 patients and identified gut microbial signatures in patients with severe COVID-19 disease and their correlation with circulating cytokine abundance, as well as gut microbial changes in response to convalescent plasma therapy (CPT) and linked to disease outcomes.

## Results

### Gut microbiota dysbiosis and disease severity in patients with COVID-19

Demographic parameters, co-morbid clinical conditions, and a few baseline clinical parameters are provided in supplementary Table 1. Given the observations that SARS-CoV-2 infection may affect the taxonomic and functional attributes of the gut microbiome, we first investigated the effect of different disease severity conditions on the faecal microbiome of patients at the baseline level. Analysis of the data obtained from sequencing of the 16 S rRNA genes generated a total of 24,590,191 reads from 84 samples, with a minimum of 119,168 and a maximum 750,883 reads after quality filtering. The average number of reads generated was found to be 292,740 (supplementary Table 2).

Since some features with very small counts in very few samples are likely due to sequencing errors or low-level contaminations, hence we have set the filtering criteria at a prevalence of 10%. The sequencing depth revealed a total of 20 phyla, 34 classes, 89 orders, 172 families, and 431 genera among the 84 samples. (Fig. 1A). The microbial composition of each patient is represented in supplementary Table 3. At the phylum level it was observed that in case of severe COVID-19 patients, relative abundance of Firmicutes was the highest (39.36%) followed by Actinobacteria (33.89%), Proteobacteria (15.15%), Bacteroides (11.08%), Cyanobacteria (0.35%) and Desulfobacterota (0.14%). The relative abundance for each of the 7 Mild patients had a higher abundance of Firmicutes (43.03%), Bacteroidota (17.59%) and Cyanobacteria (2.3%), while showing considerable lower abundance of Proteobacteria (3.38%). Considering the inconsistency in sample size (Mild=7, Severe covid=77) these results may not reflect the actual landscape of microbial composition in COVID-19 patients, with varying disease severity. The relative abundance of these top six phylum in each sample belonging to severe and mild COVID-19 patients (n=84) is represented in (Fig. 1B). At the baseline (time-point 1 or T1), the alpha diversity index (, Chao1, Shannon, Simpson and Faith PD) was calculated and



**Fig. 1** Taxonomic composition and microbial diversity in Indian patients with COVID-19 (A) Heat tree representing the organization of taxa from phylum through genus level. On the lower right-hand side is the colour scale. Each node represents a particular taxon used to classify the OTU whereas the edges represents where it fits in the taxonomy hierarchy. The nodes diameter is proportional to the number of OTUs and the width of the edge is equal to the number of reads. (B) Phylum abundance bar plot showing the relative abundance of six different phylum (Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria and Desulfobacterota) in severe and Mild subjects. (C) Alpha diversity was found to be significant for Chao index ( $p$ -value = 0.02), Shannon index ( $p$ -value = 0.004) and Simpson index ( $p$ -value = 0.01) whereas Faith PD ( $p$ -value > 0.05) was non-significant at the baseline level. The box indicates the interquartile range (IQR). The median value is represented as a line within the box and whiskers extend to the extreme value that is within 1.5\*IQR. (D) Beta diversity at baseline level: Principal coordinate analysis plot (PcoA) of the gut microbiome of severe and mild patients at the baseline level. The blue dots represent subjects with severe disease and yellow dots represent mild subjects. Both the groups represented on the ordination plot with a variance of 26.3% (Axis1) and 16.4% (Axis2) respectively. The statistical significance of variance between mild and severe patients was calculated using Permutational multivariate analysis of variance (PERMANOVA) ( $R^2=0.0652$ ,  $P$ -value=0.002)

significance was tested among the two groups as per Shapiro-Wilk test It was found that Chao1 ( $p$ -value=0.02), Shannon ( $p$ -value=0.004), Simpson ( $p$ -value=0.01) were all significantly different between severe and mild COVID-19 patients), whereas, Faith PD ( $p > 0.05$ ) was found to be non-significant (Fig. 1C, supplementary Table 4). In the case of beta diversity, it was observed that the Bray Curtis distance matrices showed a high significance difference between two groups at T1. Both

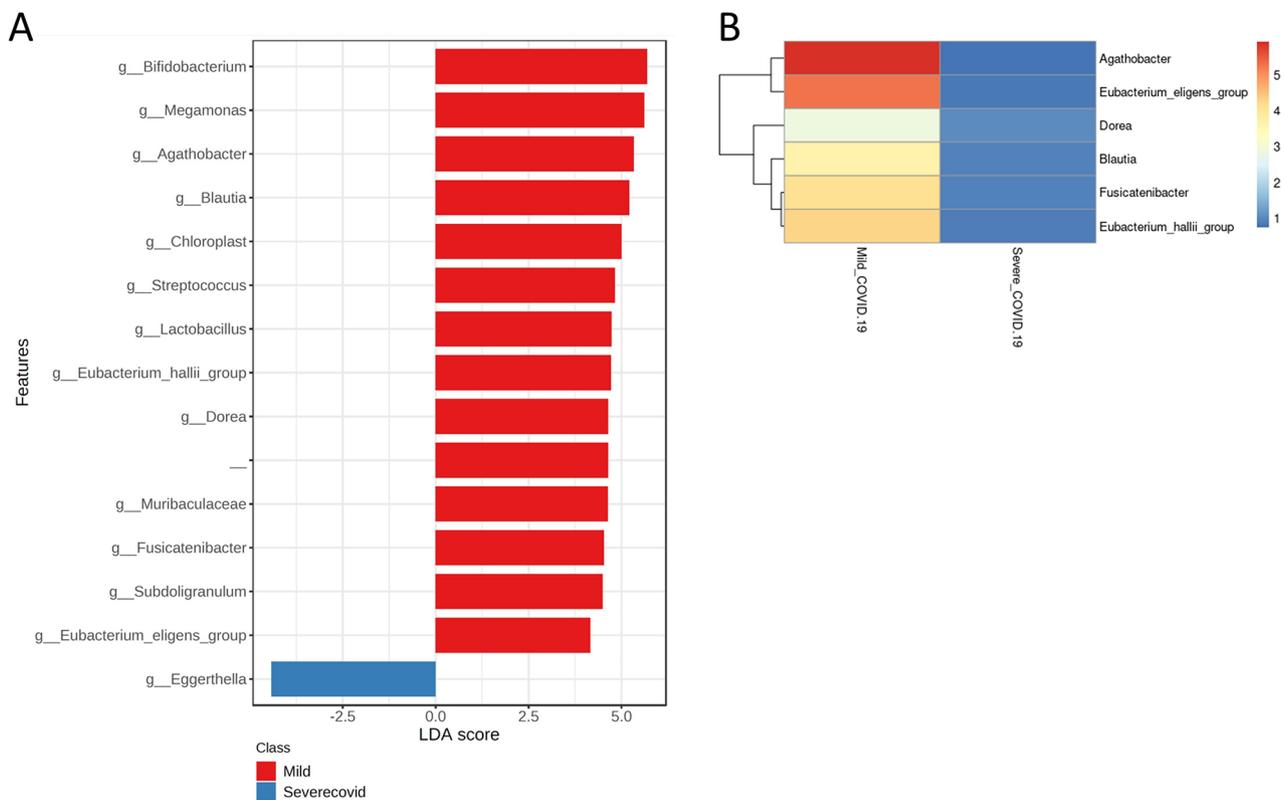
the groups are represented on the ordination plot with a variance of 26.3% (Axis1) and 16.4% (Axis2) respectively (Fig. 1D, supplementary Table 5). Statistical significance of variance was calculated using permutational multivariate analysis of variance (PERMANOVA), which reflected that severe and mild COVID-19 patients had variance among them ( $R^2=0.0652$ ,  $p$ -value=0.002).

We extended our analysis at the genus level, to identify differentially abundant bacterial genera between the

mild and severe disease groups. On performing FDR correction 15 genera were significantly different between the two groups. LEfSe analysis to compare the effect size between mild and severe COVID-19 groups yielded a histogram of the LDA scores which was computed for features that showed differential relative abundance between the two groups (Corrected p-value threshold=0.1) (Fig. 2A) Six gut commensal genera belonging to Lachnospiraceae family (phylum Firmicutes) and the genus *Bifidobacterium* (phylum Actinobacteriota) were significantly enriched in patients with mild COVID-19 disease after FDR correction, viz. *Agathobacter*, *Blautia*, *Fusicatenibacter*, *Dorea*, *Eubacterium\_hallii\_group* and *Eubacterium\_eligens\_group* (p-value<0.05, FDR corrected). (Figures 2B and 3A, Supplementary Table 6). *Eubacterium\_hallii\_group*, *Eubacterium\_eligens\_group*, *Dorea* and *Blautia* are well-known producers of the anti-inflammatory short-chain fatty acid (SCFA) butyrate, which is the primary metabolic fuel for colonocytes and important for maintaining colonic epithelial integrity

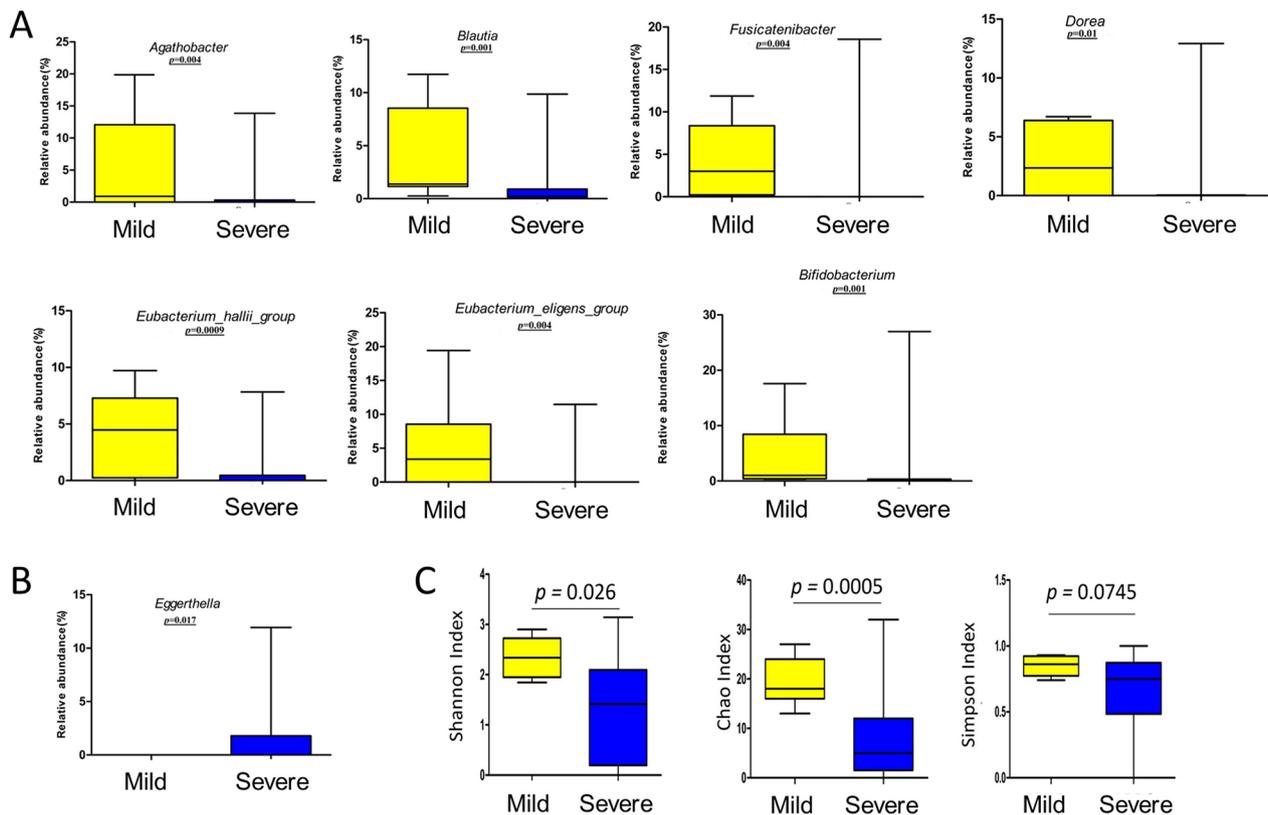
[30]. Hence, depletion of these in severe COVID-19 patients may be relevant to disease pathogenesis. Apart from butyrate, the production of other SCFAs is mediated by bacteria such as *Bifidobacterium* species (belonging to the Phylum Actinobacteria) that produce acetate and lactate during carbohydrate fermentation.

On the other hand, opportunistic pathogens, such as *Eggerthella*, was found to be significantly enriched in severe patients (p<0.05, FDR corrected) (Fig. 3B) in addition to *Enterococcus* and *Staphylococcus* which were more abundant in the severe group (p<0.05, Mann Whitney U test) (Supplementary Fig. 1) but not statistically significant after FDR correction. *Staphylococcus* is a virulent pathogen that is currently the most common cause of infection in hospitalized patients [31]. *Enterococci* are not detrimental on limited gut abundance — however transmission to other parts of the body may cause lethal infection [32]. Several studies have shown that the increased gut permeability in rheumatoid arthritis patients as well as Th17 activation in a murine colitis



**Fig. 2** Differential abundance of gut microbiome in Mild and severe COVID-19 patients from India

(A) LEfSe analysis identified the most differentially abundant genera (n=15). LDA scores > 2 are shown. – in the figure is an unidentified genus from the family Lachnospiraceae. (B) Among the 15 genera, Lachnospiraceae family dominates the list with (n=6) genera (p-value < 0.05, Mann Whitney U test, FDR corrected) The rows represent the mean relative abundance of each genus whereas the column stands for the mean relative abundance (%) of the two groups. Heatmap showing the bacterial composition (Lachnospiraceae family) of different COVID-19 patients (mild and severe COVID-19) at the genus level. The mean relative abundance of each bacterial genus is represented by the colour of the scale ranging from red (high mean relative abundance) to blue (low mean relative abundance) as depicted on the right side of the heatmap. All genera belonging to Lachnospiraceae family were enriched in mild COVID-19 patients as compared to the severe population



**Fig. 3** Relative abundance of differentially abundant genera between Mild and severe COVID-19 patients from India

(A) Box plots showing the relative abundance (%) of 6 differentially abundant genera in mild and severe COVID-19 patients from the Lachnospiraceae family ( $p$ -value < 0.05, Mann Whitney U test, FDR corrected). The relative abundance (%) of genus *Bifidobacterium* was also enriched in mild patients ( $p$ -value < 0.01, Mann Whitney U test, FDR corrected). All the above-mentioned commensal gut microbes were significantly high in mild patients than in the severe disease group. (B) Box plots showing the relative abundance (%) of the opportunistic pathogen *Eggerthella* statistically different between the two groups ( $p$ -value < 0.05, Mann Whitney U test, FDR corrected). (C) Alpha diversity in the mild and severe COVID-19 patients was computed. In Lachnospiraceae family diversity was found to be significant for Shannon index ( $p$ -value = 0.0026, Mann Whitney U test), Chao index ( $p$ -value = 0.0005, Mann Whitney U test), and non-significant for Simpson index ( $p$ -value = 0.074, Mann Whitney U test). The box indicates the interquartile range (IQR). The median value is represented as a line within the box and whiskers extend to the extreme value that is within  $1.5 \times \text{IQR}$ .

model are associated with an increased abundance of *Eggerthella* in the gut microbiota [33–35].

Since members of the Lachnospiraceae family were found to be the most differentially relatively abundant taxa between patients with mild and severe COVID-19, we compared the diversity in the Lachnospiraceae family between two groups of COVID-19 patients. Our analysis revealed that, Shannon ( $p < 0.002$ ) and Chao ( $p < 0.0005$ ) indices were statistically more prevalent in the patients with mild disease, whereas Simpson diversity did not show any statistical difference in richness and evenness between the two groups of patients ( $p > 0.05$ ) (Fig. 3C, supplementary Table 7).

Then, to gather some insight on the association between the differentially abundant genera ( $n=41$ ), we constructed the co-occurrence networks (Pearson's correlation, with a cut-off  $R \geq 0.7$ ) and compared it between patients with mild and severe COVID-19. In patients with mild disease, the genus *Dorea* from the Lachnospiraceae

family, which is a known producer of the SCFAs and butyrate [36], was found to be negatively correlated with the genera *Enhydrobacter* (Phylum Alphaproteobacteria) and *Fusicatenibacter* (Phylum Firmicutes). Besides these, the genera *Allisonella* and *Megamonas* also showed negative correlations. From these associations, we can compute that there is a strong negative correlation between butyrate and acetate-producing commensal gut bacteria and non-indigenous pathogens. Rest of the genera showed strong positive associations between them (Supplementary Fig. 2A and 2B, supplementary Table 8). In case of patients with severe COVID-19 disease, two correlative clusters were formed. The first cluster consists of 16 genera which were positively correlated with each other. The second cluster was formed by *Enhydrobacter* and *Ochrobactrum* (both belonging to Phylum Proteobacteria) showing a strong positive correlation. Both are opportunistic pathogens in the human gut causing severe infections in immunocompetent hosts [37]. From our

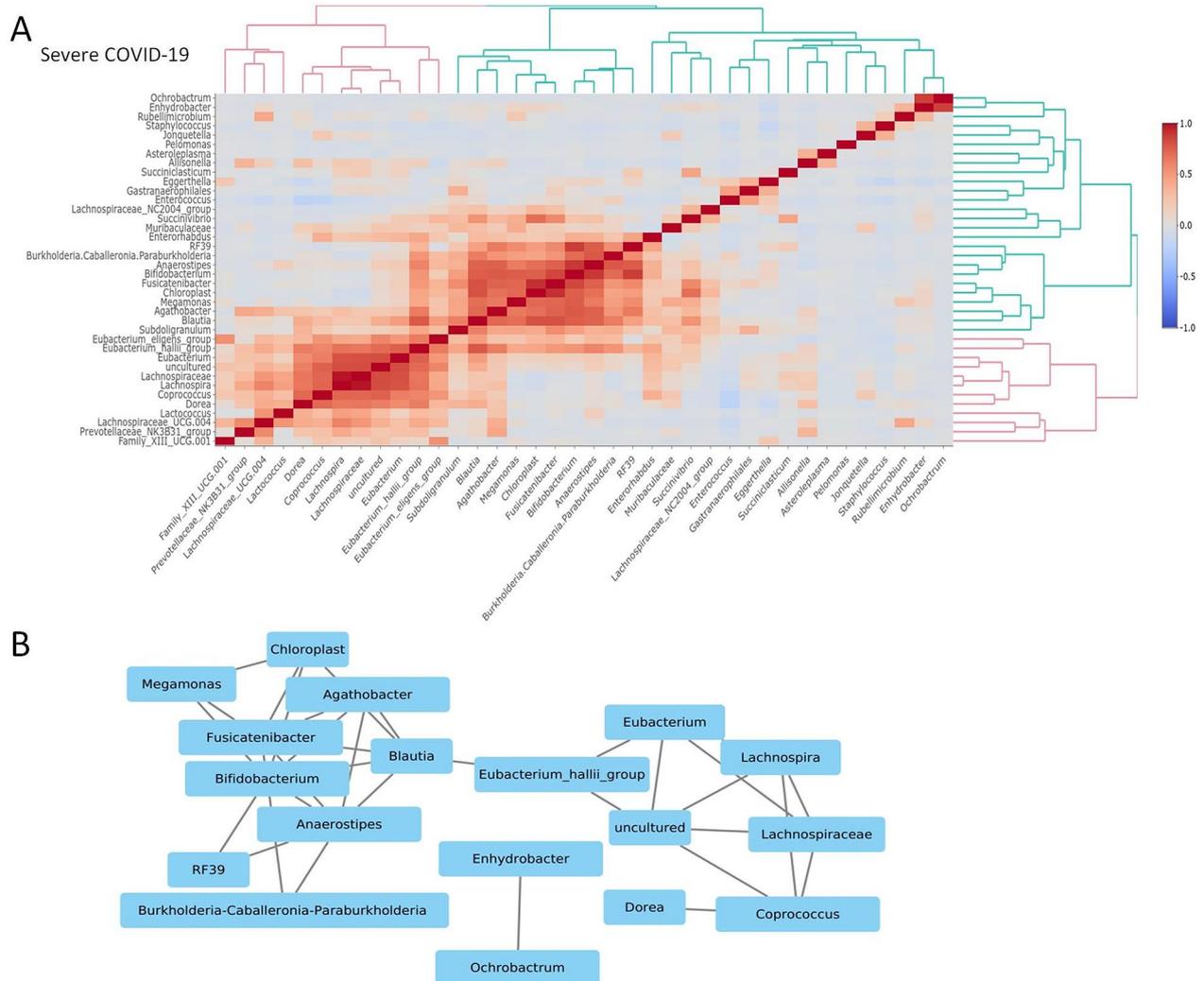
correlative analysis within the microbiome, we can infer that pathogenic microbes and beneficial gut bacteria have strong positive associations within themselves (Fig. 4A and B, supplementary Table 9).

**Genomic repertoire of the gut microbiota found depleted in severe COVID-19 is enriched for potentially immunomodulatory metabolic pathways**

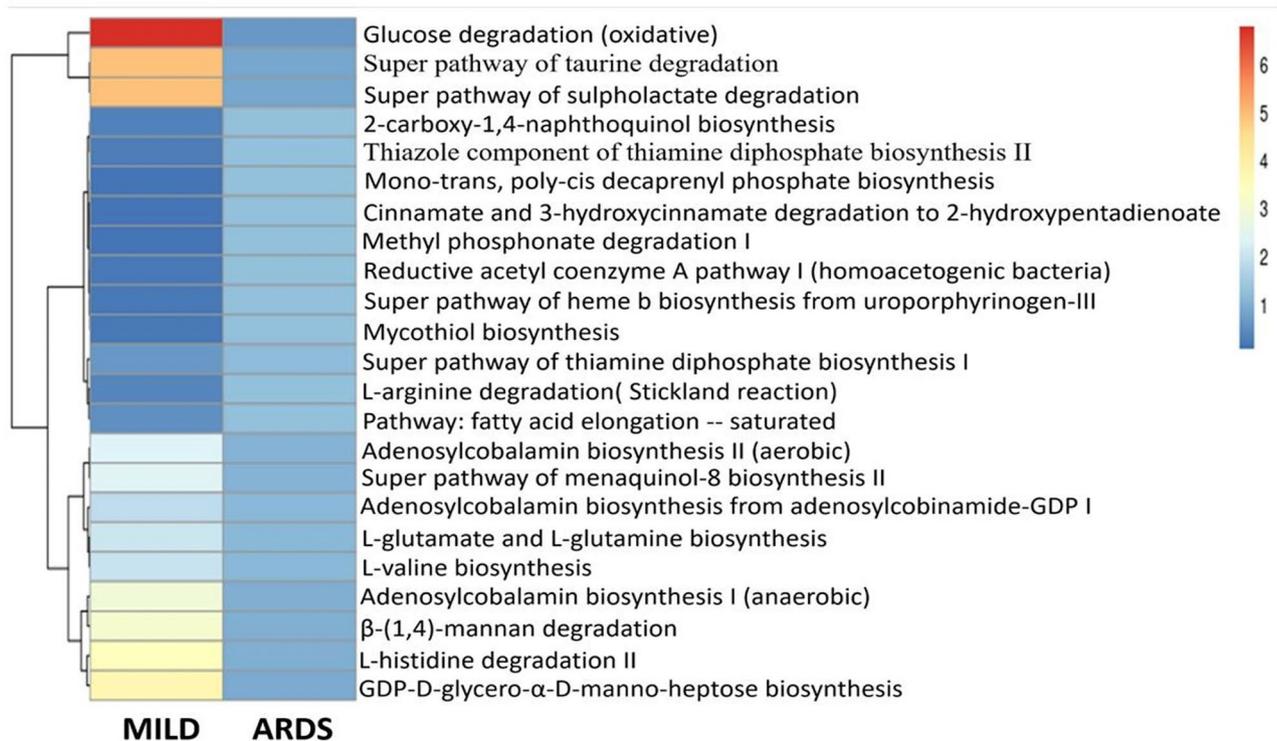
Next, the metabolic capacity of the microbiome was inferred using microbial diversity obtained from the 16 S rRNA gene amplicon data by PICRUSt2 software (supplementary Table 10). The metabolic pathways of the gut microbiome were compared between different populations. The analysis revealed the differential abundance

of 23 metabolic pathways inferred from the gut metagenome between mild and severe COVID-19 patients (p-value<0.05, Mann Whitney U test), however, these pathways were not significantly different after computing the FDR correction using Benjamini-Hochberg method (Fig. 5, supplementary Tables 10 and supplementary Table 11). Among these pathways, twelve were found to be highly enriched for the mild disease.

Mean relative abundance of the L-histidine degradation II pathway was highly enriched in the mild COVID-19 population (mean=3.46 in mild disease, mean=0.98 in severe disease, p-value<0.05, Mann Whitney U test). Catabolism of this amino acid yields glutamate and decarboxylation of histidine produces histamine.



**Fig. 4** Correlation between different gut commensals and opportunistic pathogens in severe COVID-19 patients. Co-occurrence among each pair of genera at T1 time point in the gut microbiome was calculated using Pearson's correlation coefficient (r). The relative abundance of the genera was used for calculation of correlation network. **(A)** Heatmap showing the correlation matrix between the different gut bacteria in patients with severe COVID-19. Red squares represent strong positive correlation blue squares represent strong negative correlation and white square represent non-significant correlations. **(B)** Co-occurrence network with threshold set to  $r \geq 0.7$  or  $r \leq -0.7$ ,  $p < 0.05$  between the different genera in the severe COVID-19 (n=44) group. The black lines (edges) represent strong positive interaction between the genera (signified by nodes)



**Fig. 5** Heatmap of bacterial gene functional prediction using the latest PICRUSt2 algorithm from the faecal samples of Indian COVID-19 patients. Using the amplicon sequence variants (ASV) and the biome table generated from QIIME2 database as input, PICRUSt2 predicted the KEGG-level pathways. The relative abundance was then calculated from the observed abundance of each pathway in each sample. In mild and severe COVID-19 patients a total of 23 metabolic pathways were statistically significant between the groups ( $p$ -value < 0.05, Mann Whitney U test). Among these 23 metabolic pathways, 12 were found to be highly upregulated in mild patients ( $p$ -value < 0.05, Mann Whitney U test).

Histamine is responsible for inflammation in allergic reactions in general, but bacteria-metabolized histamine has been shown to inhibit the production of pro-inflammatory cytokines such as TNF- $\alpha$  in vivo and IL-1 and IL-2 in vitro [38, 39], in addition to preventing intestinal bacterial translocation.

Several amino acid metabolism pathways were also found to be high in patients with mild disease. The mean relative abundance of L-glutamate and L-glutamine biosynthesis (mean was 1.11 for severe COVID-19, 2.06 for mild disease) and L-valine biosynthesis (mean was 1.11 for severe COVID-19, 1.99 for mild disease) pathways were found to be under-represented in patients with severe disease ( $p$ -value < 0.05, Mann Whitney U test). In fact, various amino acids produced from the microbial protein fermentation pathways can serve as precursors for SCFA synthesis. Amino acids that are metabolized to the butyrate by anaerobic bacteria include glutamate, threonine, and lysine [40].

Cobalamin B12 biosynthesis pathways (I and II) were upregulated in the mild COVID-19 patients (mean 2.35, 2.95 in mild and 1.08, 1.02 in severe disease for I and II respectively,  $p$ -value < 0.05). Vitamin B12 serves as a co-factor for nucleotide and amino acid biosynthesis and

acts as a metabolic substrate for the gut microbiome. Besides, it also provides protection against stroke in selected patients [41].

#### Association of faecal microbial entities with systemic hyper-inflammation in COVID-19 patients

The systemic hyper-inflammation characterized by a differential abundance of inflammatory cytokines in response to COVID-19 infection distinguishes patients with mild symptoms from severe [42]. In patients with severe COVID-19, a systemic hyper-inflammatory state is encountered, associated with a cytokine storm, i.e., the large abundance of pro-inflammatory cytokines in circulation [43, 44]. This in turn may lead to septic shock and multiorgan failure [45, 46]. From our previous analyses it was apparent that gut microbiome plays an important role in disease severity and several bacteria are associated with disease severity. To explore any plausible association of the microbial dysbiosis with the nature of the cytokine storm encountered in the severe COVID-19 patients in our cohort, we ran a set of correlative analyses between the gut genera (both commensal and pathogenic) apparent from the faecal DNA amplicon sequencing data and plasma concentrations of 36 cytokines.

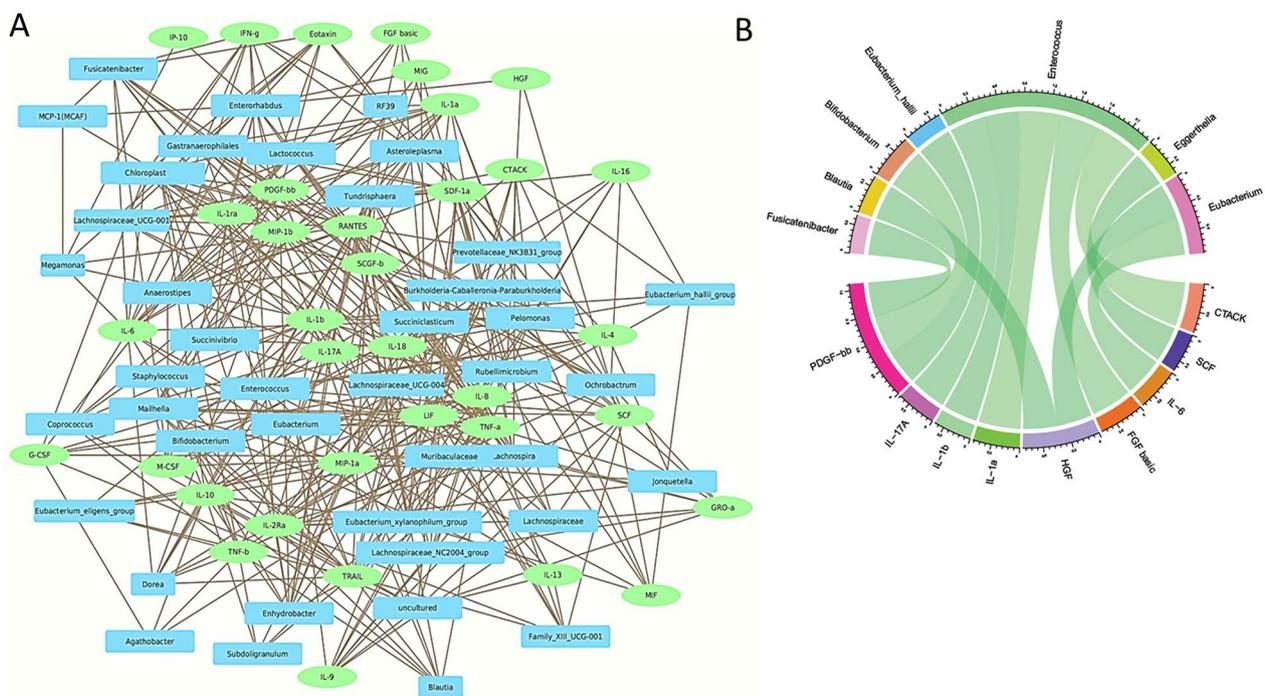
To explore the plausible association of relative abundance of faecal microbial entities with absolute plasma abundance (pg/ml) of 36 cytokines we constructed a co-occurrence network (Spearman's correlation, with a cut-off  $R \geq 0.5$ ) and compared between patients with either mild or severe COVID-19. IL-4, IL-9, IL-10, and M-CSF were discovered to be positively associated with *Lachnospira*, *Lachnospiraceae\_UCG\_004*, *Eubacterium*, *Agathobacter*, *Enhydrobacter*, and *Fuscatenibacter* (Fig. 6A, supplementary Table 12). On the other hand, pro-inflammatory cytokines like IL-6, IL-8, IL-18, G-CSF, TNF- $\alpha$  and TNF- $\beta$  were also found to be strongly correlated with several gut microbes, perhaps pointing to a feed-forward regulation. G-CSF (Granulocyte colony stimulating factor) or granulocyte production cytokine was found to be positively associated with *Agathobacter*. G-CSF, which is a growth factor that stimulates the bone marrow to produce white blood cells to reduce the risk of infection in the body, was correlated with the butyrate producing gut bacteria, which in turn is beneficial for reducing the growth of non-indigenous pathogens [47, 48].

We then checked for the association of cytokines and gut microbes in the severe COVID-19 patients. However, with correlation cut-off value of 0.5 we found that only

the genus *Enterococcus* showed a positive correlation with the CC chemokine CTACK while none of the other associations crossed this cut-off R value (data not shown), supplementary Table 13). Thus, we extended our analysis by lowering the threshold parameter to 0.4 and found significant positive associations which are represented in a circos plot in Fig. 6B. Positive correlations were observed between *Fuscatenibacter*, *Bifidobacterium* and *Eubacterium\_hallii* with PDGF-bb, *Blautia* and *Eubacterium* with HGF, *Enterococcus* with CTACK, FGF basic, IL-1a IL-1b and IL-17 A, *Eggerthella* with SCF and *Eubacterium* with IL-6.

### Gut dysbiosis in response to therapy in severe COVID-19 patients

In a fraction of the severe COVID-19 patients (n=32) faecal samples were collected 7 days post enrolment (time-point 2 or T2) and faecal DNA was analysed by 16s rRNA amplicon sequencing. We classified the patients based on whether they did or did not receive major antibiotic pharmacotherapy with or without CPT in the intervening period of one week. As reported earlier, CPT was given to the patients, randomized into the intervention arm, as two doses of 200ml of convalescent plasma



**Fig. 6** Association between gut bacteria and plasma cytokine levels in mild and severe patients

(A) The co-occurrence among the significantly different genera and cytokines was calculated using Spearman's rank correlation coefficient ( $\rho$ ) in Mild patients. The relative abundance of the genera and log fold change (absolute values) were used for the calculation and threshold was set to  $r > 0.5$ . All calculations were done using R package, function `cor()`. The nodes represent genera and cytokines which are colour encoded. Green nodes represent cytokines and the blue nodes represent genera. The edges represent strong positive interaction between cytokine and genera. (B) Circos plot representing the co-occurrence among the significantly different genera and cytokines in severe COVID-19 patients calculated using Spearman's rank correlation coefficient ( $\rho$ ) with the threshold set to  $r > 0.4$ . Only positive associations crossed the threshold cut-off and are represented in green where a darker shade represents stronger correlation.

on two consecutive days—the first dose being on the day of recruitment (T1 sampling) [49]. Plasma from donors who have recovered from COVID-19 contains neutralizing antibodies against SARS-CoV-2. In addition, a prominent anti-inflammatory effect of convalescent plasma was also reported earlier, presumably due to diverse anti-inflammatory proteins circulating in the blood in the convalescence period [50].

We checked for changes in the gut microbial composition of severe COVID-19 patients before taking convalescent plasma (T1 time point, n=15) and after administration of plasma therapy (T2 time point, n=15). Due to small sample size FDR correction, did not yield any genus to be significantly altered between the two groups (supplementary Table 14). However, the mean relative abundance (%) of the genus *Lachnospirillum* was significantly lower in the T2 time-point, i.e., post-CPT while genus *Kocuria* (phylum Actinobacteriota) and genus *Pannonibacter* (Phylum Proteobacteria) were more abundant after CPT (p-value<0.05, Mann Whitney U test) (Supplementary Fig. 3A supplementary Table 14). *Lachnospirillum* is known to negatively influence the circulating levels of acetate, besides being involved in the biosynthesis of detrimental lipid compounds [51]. Not much is known about the clinical significance and pathophysiology of genus *Pannonibacter* and *Kocuria* in the human gut, hence further research is needed to interpret these data.

We next analysed the effects of different therapies administered to patients with severe COVID-19 disease to understand the alteration of gut microbial composition. All the severe patients analyzed received antibiotic (Ab) and antiviral (Av) pharmacotherapy (n=14). Hydroxychloroquine and Remdesivir, a broad-spectrum antiviral drug, were used for treating the patients. Among the other antimicrobial drugs, different antibiotics (Ab) were also variably used, viz. Doxycycline (broad spectrum tetracycline class), Clarithromycin, Azithromycin (both belong to Macrolide class), Faropenem (Beta-lactam antibiotic), Co-amoxiclav (penicillin like Ab), Levofloxacin and Norfloxacin. First, we compared the gut microbial enrichment at T1 and T2 for patients receiving only Ab and Av therapy without any CPT (Supplementary Table 15). Interestingly, potentially pathogenic genera like *Streptococcus*, *Alloprevotella* and *Acinetobacter* (phylum Proteobacteria), an often-multidrug resistant pathogen, were enriched after these therapies at T2 timepoint. Nevertheless, a significant enrichment of the potentially metabolic health-promoting genus *Subdoligranulum* was also documented [52] (Supplementary Fig. 3B). However, observations between the two groups reflect significant mean differential relative abundance (p-value<0.05, Mann Whitney U test),

which was not evident on applying FDR correction due to the small sample size (Supplemental Table 15).

As we also had this sub-group of patients who also received CPT in addition to the Ab and Av therapy (all therapy, n=13), we explored the differential gut microbial enrichment at T2 between these two groups. As mentioned previously the sample size did not favour significant differences on FDR correction (Supplementary Table 16). However, on analysing the mean differential relative abundance between the two groups four genera belonging to the phyla Bacteroidota, Proteobacteria and Firmicutes were found to be differentially abundant at T2 between the patients receiving only antibiotic/antiviral therapy and those also receiving CPT in addition (p-value<0.05, Mann Whitney U test). The relative abundance (%) of the genus *Acinetobacter* (phylum Proteobacteria), an often multidrug-resistant pathogen, was found to be high at T2 in patients who did not receive CPT (p-value<0.05), whereas the relative abundance (%) of the genera *Prevotella* (phylum Bacteroidota), *Acidaminococcus* and *Negativicoccus* (both from Phylum Firmicutes) were enriched at T2 in patients also receiving CPT (p-value<0.05, Mann Whitney U test) (Supplementary Fig. 3C, supplementary Table 16). Whether CPT had any role in the significant decline in the relative abundance (%) of *Acinetobacter* in the gut warrants further studies in larger cohorts of patients.

We next analysed the gut dysbiosis at T2 among the patients taking single (n=7), double (n=10) or multiple combinations of antibiotics (n=10). Altogether, we found five significantly different genera between these three groups. The relative abundance % of the genus *Holdemanella* from the phylum Firmicutes decreased as the more antibiotics were combined (p-value<0.05, Kruskal Wallis H test). *Holdemanella* (formerly *Eubacterium bififormis*) produces SCFAs and long chain fatty acid (LCFA) 3-hydroxyoctadecaenoic, and has been earlier reported to induce anti-cancer and anti-inflammatory effects [53]. The relative abundance of genus *Aerococcus* and *Brevundimonas* from the phylum Proteobacteria increased in patients taking more than two antibiotics (p-value<0.05, Kruskal-Wallis H test). *Brevundimonas* was previously reported to be either hospital or community-acquired and resistant to all second and third generation antibiotics [54]. On the other hand, members of Bacteroidetes (genus *Parabacteroides*) and *Lachnospirillum* (family Lachnospiraceae) were found to be more abundant in patients taking two antibiotics, whereas it decreased in the gut on taking more than two antibiotics (p-value<0.05, Kruskal-Wallis H test) (Supplementary Fig. 3D, supplementary Table 17). Due to low sample size in each group, the FDR corrected values were highly conserved (>0.05) and hence large cohort size is desirable for significant results. Thus, our analyses

revealed a significant effect of antibiotic use on the differential composition of the gut microbial ecology, driving over-abundance of drug-resistant and pathogenic bacteria with multiple antibiotic usage.

#### **Predicted metabolic pathway enrichment in response to therapy in severe COVID-19**

Next, we explored the modulation of the inferred metabolic capacity of the microbiome using microbial diversity obtained from the 16 S rRNA gene amplicon data in response to different therapies. First, we performed predicted change in the gut microbial functionality prediction in severe COVID-19 patients who received CPT (Supplementary Table 18). Our analysis revealed 9 differentially abundant pathways in the gut microbiome of patients before (pre-CPT, T1) and after (post-CPT, T2) convalescent plasma therapy (Fig. 7A, supplementary Table 19). The mean relative abundance of amino acid degradation pathways involving L-histidine (mean=1.82 pre-CPT at T1, mean=4.79 post-CPT at T2) and L-valine (mean=0.00 pre-CPT at T1, mean=6.66 post-CPT at T2) were found to be higher in patients after convalescent plasma therapy ( $p$  value<0.05, Mann Whitney U test). Various bacteria degrade proteinogenic amino acid to use them as a source of energy and nutrients [55]. Ergothioneine biosynthesis I (EGT) metabolic pathway associated with the gut microbiota was found to be under-represented before plasma therapy, whereas it showed a significant upregulation on CPT ( $p$  value<0.05, Mann-Whitney U test). The function of ergothioneine (EGT) in the microbial cells is not well understood even though it is believed that EGT may protect the cells from oxidative stress [56]. Methanol oxidation to carbon dioxide, representing detoxification, was also found to be abundant after taking the plasma therapy ( $p$  value<0.05). Then we extended these analyses to severe COVID-19 patients who did not receive CPT and were treated only with Ab/Av therapy (Supplementary Table 20). Our analysis revealed 13 differentially abundant pathways in the gut microbiome of patients before (T1) and after (T2) 7 days of therapy with antibiotics and antivirals (Fig. 7B, supplementary Table 21).

We then compared the inferred metabolic pathway enrichment among the gut microbes between patients receiving only antibiotic and antiviral drugs (non-CPT sub-group) and patients receiving CPT in addition to along with antibiotics and antivirals (Fig. 7C, supplementary Tables 22 and 23). Carbohydrate and amino acid degradation pathways were highly represented in the gut microbiota of patients who took only antibiotic and antiviral drugs ( $p$  value<0.05, Mann-Whitney U test). Besides these, the pathway for NAD biosynthesis (Nicotinamide adenine dinucleotide) was also found to be enriched in the non-CPT sub-group. This is the most

important coenzyme in cellular redox reactions and is primarily involved in transcriptional regulation system [57]. Interestingly, mean relative abundance of the gut microbiome associated pathways involved in carbohydrate fermentation to SCFA was also higher in the non-CPT group compared with patients also receiving CPT ( $p$  value<0.05, Mann-Whitney U test). However, on statistical computation none of these pathways had FDR corrected values<0.05.

#### **Faecal microbial signature linked to disease outcomes in severe COVID-19 patients**

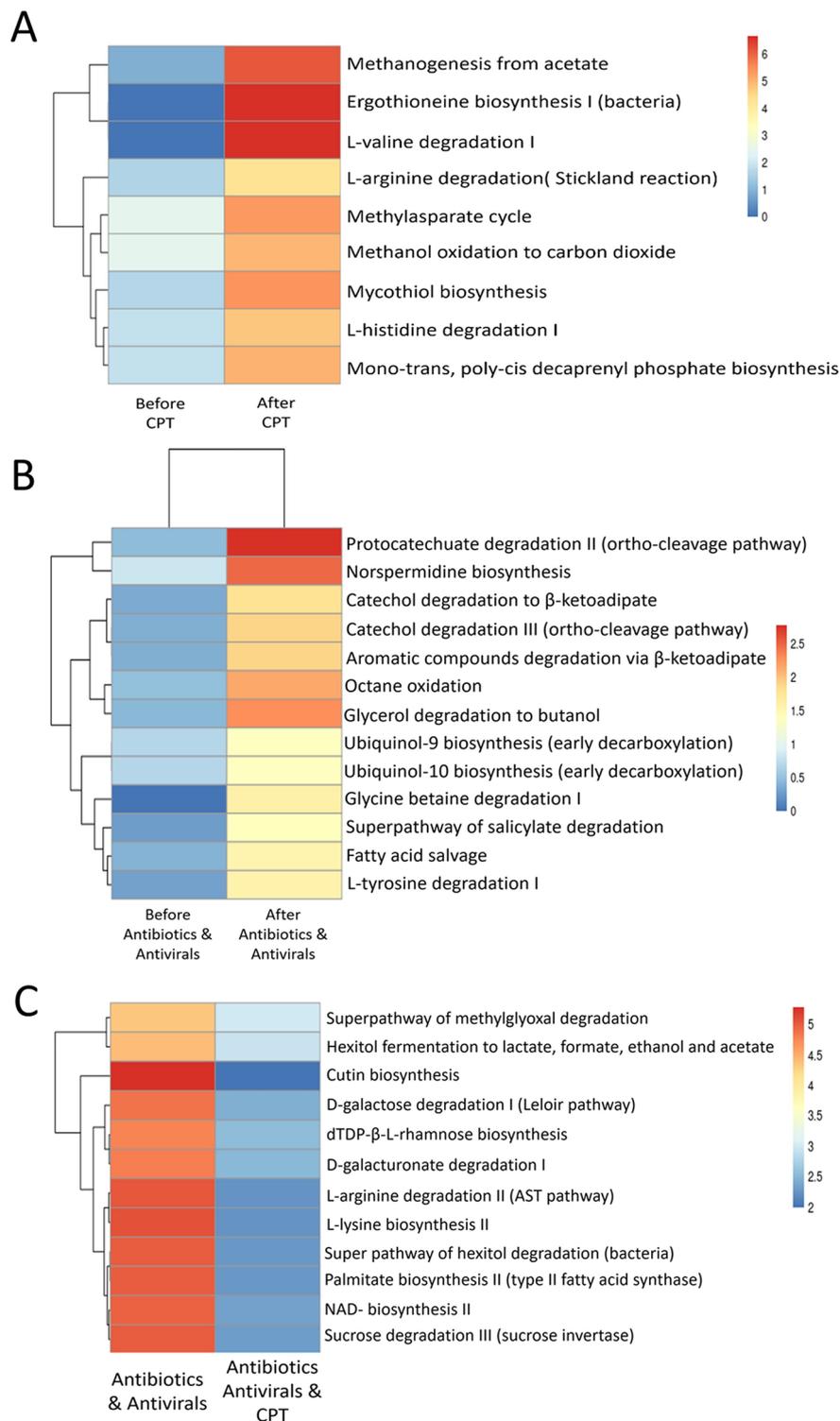
Finally, to explore if the baseline contexture (on recruitment at T1) in the faecal microbial dysbiosis was linked with the eventual clinical outcomes in the severe COVID-19 patients, we stratified the severe COVID-19 cohort based on eventual remission ( $n=31$ ) or non-remission (death,  $n=11$ ) and ran a set of comparative analyses on their T1 genus-level gut microbiome data.

We found that the relative abundance of genus *Hungatella*, a member of the phylum Firmicutes was significantly over-represented in patients who eventually succumbed to their severe COVID-19 disease ( $p$  value<0.01, Mann Whitney U test) even though this was not evident after FDR correction. (Supplementary Fig. 3E, supplementary Table 24). *Hungatella hathewayi*, from the Lachnospiraceae family, is also known as *Clostridium hathewayi*. Though it is a part of the normal gut flora, it has the potential for causing bacteraemia and sepsis [58].

We computed the alpha diversity indices at the OTU level, but none of the indices showed a significant difference (Shannon, Chao1 and Simpson,  $p$ =value>0.05) between the two groups (Supplementary Fig. 4A, supplementary Table 25). When we compared the alpha diversity indices within the Lachnospiraceae family, within the severe COVID-19 patients eventually achieving remission versus non-remission, no significant difference in Shannon, Chao1 and Simpson indices ( $p$ -value>0.05) between the two groups were observed ( $p$ -value>0.05, Mann Whitney, U test) (supplementary Fig. 4B, supplementary Table 25). Comparative analyses of the inferred functional profiling of the gut microbial ecology between these two patient sub-groups also failed to discern a significant difference in enrichment of any metabolic pathway ( $p$  value>0.05, Mann Whitney U test).

#### **Discussion**

The COVID-19 epidemic has raised awareness of the importance of life and public health worldwide. This is the first study that shows the composition of gut microbiota in an Indian cohort of COVID-19 patients and its association with disease severity and therapy outcomes. The gut dysbiosis in patients with acute respiratory distress syndrome (ARDS) is characterised by opportunistic



**Fig. 7** Relative abundance of metabolic pathways in response to different treatment regimes used for the treatment of COVID-19 in a cohort of Indian patients

(A-C) Heatmap of bacterial gene functional prediction using the latest PICRUSt2 algorithm from the faecal samples of patients at (A) T1 (Before CPT) and T2 (After CPT) time point respectively. 9 metabolic pathways were differentially abundant between the two groups. ( $p$ -value  $< 0.05$ , Mann Whitney U test). (B) 13 metabolic pathways were differentially abundant between severe COVID-19 patients ( $n = 14$ ) at T1 time point (Before antibiotics & antivirals) and T2 time point (After antibiotics & antivirals) therapy. ( $p$ -value  $< 0.05$ , Mann Whitney U test). (C) 12 metabolic pathways were differentially abundant between patients given antibiotic & antiviral therapy ( $n = 14$ ) and the ones with antibiotic & antiviral and convalescent plasma therapy ( $n = 13$ ) at T2 time point. ( $p$ -value  $< 0.05$ , Mann Whitney U test)

pathogen enrichment. Several studies have shown that respiratory viral infections are associated with gut dysbiosis. For example infection by influenza and respiratory syncytial viruses has been reported to alter gut microbial composition [23]. *Staphylococcus* which is considered to be a major human opportunistic pathogen, is a prevalent cause of morbidity and mortality worldwide, known to be associated with bacteraemia, indicating susceptibility for disease severity due to potential secondary infections [59]. On the other hand, we found an increased proportion of potential pathobionts belonging to the Enterococaceae family (genus *Enterococcus*) in patients with severe COVID-19. The presence of enterococcal strains in the gut microbiome of critically ill COVID-19 patients, may act as a pool of opportunistic and antibiotic-resistant pathogens, leading to a pro-inflammatory environment in the gut [21].

In addition to this, severe COVID-19 patients showed a depletion in the relative abundance of several gut commensals from the dominant family of Lachnospiraceae along with the genus *Bifidobacterium*. These are commensal gut bacteria which produce microbial metabolites by fermenting carbohydrates and have an important role in human metabolic and immunological homeostasis [60]. The analysis of bacterial richness and alpha diversity among the groups showed that patients with mild COVID-19 disease had higher microbial diversity than patients with ARDS and severe disease. Consistent with our results it was previously shown that there was significant depletion in the diversity of the gut microbiota in several respiratory viral infections [23]. Changes in the gut is often of fundamental importance in the pathogenesis and progression of diseases such as diabetes, obesity, autoimmune diseases and diseases related to ageing [61–63]. Patients with these diseases are established to constitute a high-risk group for developing severe COVID-19 and the associated gastrointestinal symptoms in these patients may be indicative of COVID-19 complications [7].

Convalescent plasma therapy trials for the treatment of hospitalized patients in an attempt to ameliorate disease progression have been recognized worldwide [64]. Here we had the opportunity of analysing the changes in the gut microbiome of patients before and after convalescent plasma therapy to understand whether gut microbes could be playing any role in the disease outcome. To the best of our knowledge, these data are the first of their kind in this domain. Genus *Lachnoclostridium* was found to be depleted in the gut after convalescent plasma therapy, whereas genus *Kocuria* from Mirococacceae family, which is now considered to be a human pathogen, was found to be elevated after plasma therapy. A high abundance of the genus *Prevotella* was found in patients given all therapy. Classically, *Prevotella* strains are considered

to be commensal bacterium since it is extensively present in the healthy human body, but a few strains have now been reported to give rise to opportunistic infections including pneumonia [65]. On the contrary, multidrug-resistant *Acinetobacter*, an opportunistic pathogen was relatively high in patients without convalescent plasma therapy. Furthermore, the gastrointestinal (GI) tract colonization has been linked to the development of antibiotic resistance of *A. baumannii*, presumably due to the close proximity of the organism to the enormous numbers and varieties of bacteria present [66]. The increase or decrease in the abundance of certain taxa may not be due to treatment regime alone, it might be outcome of natural course of the disease and other factors like use of dietary changes, stress, environmental exposures, etc.

The predicted metagenome analysis using PICRUSt 2 depicted metabolic pathways that were present in the metagenomes of mild and severe COVID-19 patients. Gut microbiota and its metabolites have an important role in host physiology and maintenance of immune homeostasis. Thus, from our findings, we decoded several pathways related to vitamin B12 biosynthesis, amino acid biosynthesis, amino acid degradation, and carbohydrate degradation were enriched more in mild COVID-19 patients, compared to patients with severe disease. Previous reports suggested that the functional output of the gut microbiome, mainly SCFAs and amino acids, which are considered to be important bacterial metabolites, plays a vital role in host physiology [67]. Cobalamin (Vitamin B12) has several functions including nucleotide synthesis, metabolism of long chain fatty acids and also cell metabolism, thus making it a vital factor in promoting resilience to stroke severity [41]. Decarboxylation of histidine to histamine by gut microbes has already been reported. This amino acid has potent immunomodulatory effects through activation of histamine receptors and the authors have stated that *L.rhamnosus* exerted anti-inflammatory effects on histamine signalling pathway activation [68].

Progression of SARS-CoV-2 infection to advanced stages is usually accompanied by a vicious inflammatory response that in the future often leads to multiorgan failure. Here we have shown that the relative abundance of gut microbiota in COVID-19 patients is associated with the concentration of several blood plasma cytokines, chemokines, and inflammatory markers which thereby affect disease severity and outcomes. In a recent study, it has been reported that depletion of gut microbiota in COVID-19 cohort was linked with increasing concentrations of TNF- $\alpha$ , CXCL10, CCL2 and IL-10 indicating that these taxa might have a role in reducing the ARDS associated cytokine storm [69].

This study presents the first data on an Indian cohort of COVID-19 patients to examine the association of gut

microbes with COVID-19 severity. One approach that can promote a healthy microbiome includes measures to increase the production of butyrate through the microbial fermentation of dietary fibres, which can mitigate the inflammatory milieu in the gut as well as systemically. Our findings highlight the fact that, along with standard therapy, modulation of the gut microbiome composition and functions with diets or other interventions may reduce the disease severity and could be a potential therapeutic opportunity for treating COVID-19 disease and its related symptoms.

Our study has a few limitations, including a relatively small sample size with mild disease, a lack of asymptomatic patients, and a non-COVID control group from the same hospital at the same time, which may have overlooked potential confounding factors. Although a large cohort size would have been desirable to firmly establish a relationship between COVID-19 severity and gut dysbiosis, the taxonomic dysbiosis identified in the present study is significant. The microbial richness and abundance differences between different taxa in the gastrointestinal tract are fairly high, so implementing the false discovery rate (FDR) for a limited sample size (<10) with such complexity is not recommendable. In addition, the functional potency of the targeted metagenomes was predicted based on the distribution of metabolic pathways in the core genome of similar OTUs reported elsewhere but not on the basis of study samples.

## Methods

### Subject recruitment and sample collection

The patient cohort with severe COVID-19 disease (diagnosed with ARDS) was originally recruited (May, 2020 to October 2020) in a randomized control trial (RCT) for convalescent plasma therapy (CPT) in severe COVID-19 disease (CTRI/2020/05/025209), completed and reported elsewhere [49]. Patients with mild COVID-19 disease were also recruited concomitantly at the same hospital during the same time-period. The studies were done with approval of the institutional ethics committees of ID & BG Hospital (IDBGH/Ethics/2429) and CSIR-Indian Institute of Chemical Biology, Kolkata, India, in accordance with the Helsinki Declaration. Written informed consents were taken from all subjects.

### Collection of faecal samples and DNA isolation

Faecal samples were collected from 7 mild patients and 45 severe patients (Details in supplementary Table 1) immediately after enrolment (designated time-point 1 or T1) and from 32 of these severe patients (SOC n=16, CPT n=16), follow up faecal samples were collected 7 days post enrolment (designated time-point 2 or T2) to understand changes in the gut microbial composition between different diseased states and in response to CPT.

The faecal samples were collected in 5ml 95% ethanol in PBS and stored at -20°C until DNA isolation. For faecal DNA isolation, first the sample vials were centrifuged at 10,000 g for 30 min and then the supernatant was discarded. The pellet was then hydrated with C1 solution of the Qiagen DNeasyPowerSoil Kit (Hilden, Germany) and the solution was then transferred to the PowerBead Tubes and the standard protocol of the kit was followed accordingly. The extracted DNA samples were stored at -20°C until being sent for sequencing.

### Faecal DNA amplicon sequencing

Amplicon-based sequencing of the V3-V4 hyper-variable region of 16S rRNA gene with the faecal DNA samples was done by MedGenome Labs Ltd, Bangalore, India. In brief, the V3-V4 hyper-variable region of 16S rRNA gene from the isolated DNA, was amplified using universal barcoded primer pairs: V3V4F (5'CCTACGGGNGGCWGCAG'3) and V3V4R (5'GACTACHVGGGTATCTAATCC'3) [70]. Quantification of the DNA samples was done using Qubit DNA HS Assay. The DNA samples were diluted to 5ng and were amplified for 16S rRNA gene (~1.5 KB) using the universal 16S forward and reverse primers with a positive control and no template control in a step-up strategy by giving 35 PCR cycles. All 16S PCR products were further used to amplify V3-V4 region (~465 bp) using specific primers. The amplified V3-V4 PCR products were then cleaned up (1X) using AgencourtAM Pure XP beads (Beckman Coulter) so that the non-specific fragments get removed before proceeding with library preparations. The cleaned V3-V4 product was taken for library preparation using NEBNext Ultra DNA Library Prep Kit for Illumina (NEB). All the prepared libraries were checked for fragment distribution on Fragment Analyzer using HSNGS Fragment Kit (1-6000 bp). For this, the amplicon was end repaired and in a single enzymatic reaction it was mono-adenylated at 3'. The next step was ligation of the DNA fragments with NEB hairpin-loop adapters in a T4-DNA ligase-based reaction. Following ligation, USER enzyme (a combination of UDG and Endo VIII) was used for linearizing the loop containing Uracil, to make it available as a substrate for PCR based indexing in the next step. During PCR, barcodes were incorporated using unique primers for each of the samples thereby enabling multiplexing. Quantification of the prepared libraries were done using Qubit HS Assay. The qualified libraries were then sequenced on Illumina Miseq instrument to generate 0.5 M, 250BP paired end reads. All the raw sequences generated were further analyzed for taxonomic classification.

### Analyses of faecal microbiome data

The raw reads were quality controlled with Trimmomatic version 0.39 [71]. It removes adaptors and trims low

quality bases from the 3' and 5' end of reads to generate decontaminant raw reads. It also discards trimmed reads if their length is less than 60nt. Quantitative Insights into Microbial Ecology (QIIME2, version 2018.11), a custom pipeline was used to process and analyse decontaminant raw reads [72]. Next, DADA2 package was used to demultiplex and join the paired end reads to generate long sequence [73]. This package infers exact amplicon sequence variants (ASVs) from high throughput amplicon sequencing data. Then, each sequence was assigned its taxonomy by using a pre-trained Naive Bayes classifier [74] by searching in Silva ribosomal RNA (rRNA) database release-138 [75] at 97% sequence similarity. Based on the sequence data, the taxonomy can be defined at different levels of resolution (phylum, class, order, family, genus, and species). The primary microbiome analysis was done in Marker Data Profiling (MDP) in Microbiome analyst [76, 77]. The relative abundance (%) was computed for each taxonomical hierarchy in the R version 4.1.3. The alpha diversity indices (Shannon or Shannon-Weaver, Chao1 and Simpson) were calculated using the index function present in 'vegan' ecological package integrated in R software [78]. The faith PD was calculated using QIIME 2 [72]. There is no general agreement as to which is the best diversity index for microbial community diversity, however Shannon and Simpson indices have been recommended to actively measure microbial diversity [79, 80]. Here in, we describe the estimates of species richness and evenness in the study of microbial communities.

#### Co-occurrence network

In order to identify taxonomic groups having positive or negative influence on COVID-19 severity, pairwise correlations between the relative abundances of the commensals and pathogenic genera were computed using Pearson correlation coefficient ( $r$ ) in patients with mild and severe disease separately. Similarly, co-occurrence between genera abundance and the log fold change of plasma cytokine in patients were calculated using Spearman's rank correlation ( $r_s$ ). The threshold was set to  $r > 0.7$ , for the different genera in mild ( $n=7$ ) and severe disease ( $n=44$ ) conditions, and to  $r > 0.5$ , for the complete set of cytokines and differentially abundant genera in mild and severe disease separately. Cytoscape plugin Co Net was used for the construction of microbial co-occurrence network (<http://apps.cytoscape.org/apps/conet>) [81]. It is an available network construction method by which microbial co-occurrence are detected by putting together various association methodologies simultaneously and finally resulting in a consensus network. Wherein, the nodes represent genera and cytokines and the edges represent the, most significant association between the nodes [82].

#### Predictive functional analysis

Functional predictions of the bacterial communities from the gut metagenome were computed through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States using the latest PICRUSt 2 v2.4.2 software, based on the 16 S rRNA marker using the (<https://github.com/picrust/picrust2/wiki>) as described by Douglas et al. in 2020 [83]. The input used was based on the unique amplicon sequence variants (ASVs) and the Biome file generated from the QIIME 2 database. Each ASVs were represented with a unique feature ID in the input files. In the first step HMMER (<http://www.hmmerr.org/>) was used for the multiple assignment of the exact sequence variants (ESVs). The ESVs were placed in the reference tree with evolutionary placement-ng (EPA-ng) [84] and Genesis Applications for Phylogenetic Placement Analysis (gappa) omics [85] were used. A default castor R package was used for the prediction of gene families [86]. Metagenome\_pipeline.py [87] was used for metagenome prediction and KEGG (Kyoto Encyclopaedia of Genes and Genomes) database was used for comparison of the output features [88]. Finally, pathway level abundances were predicted with pathway\_pipeline.py function assigning EC numbers to MetaCyc reactions and KO abundances in KEGG pathways [87].

#### Plasma cytokine analysis

The plasma cytokine panel data, used in integrated analysis with faecal microbiome data, were generated from cryostored plasma samples, collected at recruitment, from the cohort of mild ( $n=7$ ) severe COVID-19 patients ( $n=44$ ), as previously published [49]. Multiplex cytokine analyses were done on Bioplex 200 (Biorad, Hercules, California). Out of total 48 cytokines assayed, 36 analytes, detectable in at least 70% of patients in the cohort, were finally analysed.

#### Statistical analyses

The 16S rRNA gene sequences were used to characterise bacterial community composition of each sample at the genus level. The heatmap was created using Pheatmap package in R. Differences in Shannon, Chao1 index between the 2 groups were analysed by t-test (as per Shapiro-Wilk test). Whereas Simpson and FaithPD index between two groups was computed using non-parametric Wilcoxon test (as per Shapiro-Wilk test). For pairwise comparison between the groups Wilcoxon test and for more than two groups Kruskal-Wallis H test in R were used respectively. The p-values were corrected using Benjamini Hochberg method (FDR) in R environment. Intergroup differences at the genus level were analysed by linear discriminate analysis (LDA) effect size method (LEfSe) method [89]. LEfSe uses the two-tailed nonparametric Kruskal-Wallis test to evaluate the significance of

differences in OTUs in 2 groups. Box plots were generated in R (v4.1.3) and GraphpadPrism5. Pearson's ( $r$ ) and Spearman's rank correlation ( $r_s$ ) coefficient were calculated using the functions `cor()` or `cor.test()` in the R 4.1.3 environment. The statistical significance was defined as a  $p$  value  $< 0.05$ .

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13099-023-00546-z>.

Supplementary Material 1

Supplementary Material 2

### Author contributions

D.T. and P.B. performed data analyses; Y.R., S.R.P. collected samples and clinical data; J.S., R.D. and S.C. helped in biological data acquisition and collation; A.L. and D.B. helped with fecal sample processing; A.L., S.D., D.B. participated in data analyses; B.D. supervised microbiome data analyses; D.G. designed the experiments, acquired funds to perform it and supervised the study.

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### Data Availability

The 16S rRNA gene sequences generated in this study has been submitted in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) portal and the sequences will be available in the BioProject: PRJNA895415 (Submission ID: SUB12205249) after acceptance of the manuscript.

### Declarations

#### Ethical approval

The studies were done with approval of the institutional ethics committees of ID & BG Hospital (IDBGH/Ethics/2429) and CSIR-Indian Institute of Chemical Biology, Kolkata, India, in accordance with the Helsinki Declaration. Written informed consents were taken from all subjects.

#### Conflict of interest

Authors declare absence of any conflict of interests.

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